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Validated electrospray liquid chromatographic–mass spectrometric assay for the determination of the mushroom toxins α- and β-amanitin in urine after immunoaffinity extraction

Hans H. Maurer*, Christian J. Schmitt, Armin A. Weber, Thomas Kraemer

Department of Toxicology, Institute of Pharmacology and Toxicology, University of Saarland, D-66421 Homburg (Saar), Germany

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Abstract

Specific detection of amanitins in body fluids is necessary for an early diagnosis of an intoxication with amanita mushrooms. In this paper, a liquid chromatographic-mass spectrometric assay after immunoaffinity extraction (IAE-LC–MS) is described for the determination of α- and β-amanitin in urine. The method has been validated according to the criteria established by the Journal of Chromatography B. The assay was found to be selective. The calibration curves for α- and β-amanitin were linear from 5 to 75 ng/ml. Intra- and inter-day accuracy and precision were inside the required limits. Amatoxins in frozen urine samples or immunoaffinity extracts were stable for more than 6 months, and the IAE columns could be used more than fifty times without remarkable loss in performance. LOD for α- and β-amanitin was 2.5 ng/ml and LOQ for both was 5.0 ng/ml. The absolute recoveries of α- and β-amanitin were 63% and 58% for the low quality control and 61% and 57% for the high quality control. The absolute recovery for the internal standard γ-amanitin methyl ether at 25 ng/ml was 60%. The analysis of 5 authentic urine samples from patients intoxicated by amanita mushrooms showed a good correlation between the results measured by radioimmunoassay and the IAE-LC–MS assay. A partial validation showed that the assay was also suitable for plasma analysis.

Keywords: Validation; Amanitin

1. Introduction

Amatoxins are the sole cause of fatal human *Amanita* intoxications. These mycotoxins occur in the green and white *Amanita* species and are also found in the small *Lepiota* and in *Galerina* species [1]. *Amanita phalloides* causes the majority of mushroom intoxications; it has been estimated that 10–40% of these lead to death [2]. Amanita mushrooms contain the amatoxins α-, β-, γ- and ϵ-amanitin, amanin and amanullin together with phallotoxins and virotoxins. However, the lethality is mainly attributable to the amatoxins, potent inhibitors of RNA polymerase II, especially in hepatocytes.

Specific and fast detection of amatoxins in body fluids is necessary for an early diagnosis of an intoxication entailing a large scale of invasive and expensive therapy (e.g. liver transplantation). Urine

*Corresponding author. Tel.: +49-6841-166-050; fax: +49-6841-166-051.

E-mail address: hans.maurer@med-rz.uni-saarland.de (H.H. Maurer).

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is the most important sample material for the determination of amatoxins. It has to be taken into consideration, that patients usually arrive at the hospital 12 h after ingestion of the mushrooms, since the symptoms appear after a corresponding lag time. At that time, the amatoxins are already eliminated from plasma. In case of early admission to the hospital, the amatoxins can already be detected in urine 90 min after ingestion [3].

Dorizzi et al. [4] have critically reviewed methods published for the determination of amatoxins in biological matrices. GC–MS, the gold standard in analytical toxicology [5,6], cannot be used for determination of the amatoxin peptides. HPLC methods are suitable for separation of the amanitins. However, ultraviolet (UV) or electrochemical (EC) detection required a complex protocol of extraction and purification steps often followed by column switching techniques. Defendenti et al. used HPLC–EC for the determination of α-amanitin in urine [7] after a simple solid-phase extraction (SPE). However, as for all HPLC–EC methods, this one suffered from the same disadavantages like insufficient specifity and lack of long-time stability of detection (electrode-fouling). To our best knowledge, HPLC–UV or –EC methods are not in use for the routine analysis in emergency cases of intoxications with amanitins.

The method of Brueggemann et al. for the analysis of α- and β-amanitin in urine by capillary zone electrophoresis with photodiode array detection [8] has a limit of detection of 1 μg/ml. The authors stated, that this value barely touched the clinically relevant range, which is 10–100 times lower.

Determination of amanitins in urine is possible by a commercially available radioimmunoassay (RIA) but the tracer is only stable for 1–2 months. Since the assay is only available during the mushroom season, ingestion of amanita mushrooms which were stored deep frozen cannot be monitored throughout the year. Moreover, it should be kept in mind, that the RIA result should be confirmed, because interferences may occur [4,9].

Liquid chromatographic-mass spectrometric (LC–MS) procedures can fulfill requirements of sensitivity, accuracy and specificity together with rapidity and operative simplicity for detection of α- and β-amanitin in biosamples [10,11]. We have previously developed such a LC–MS method after a simple solid-phase extraction (SPE) procedure [12]. However, during validation of the method, problems arose from urine matrix compounds co-chromatographing with the analytes and thereby disturbing the detection of α- and β-amanitin by hampering their ionization in the electrospray chamber. This phenomenon has also been described for other compounds [13]. The interfering matrix could neither be removed by modifications of SPE nor by modifications of the chromatographic system. Therefore, we tried to overcome these problems by developing an immunoaffinity extraction (IAE) procedure for selective isolation of the amanitins from urine followed by gradient elution and electrospray LC–MS detection. In the following, this new IAE-LC–MS assay is presented. It has been validated according to the criteria established by the Journal of Chromatography B [14]. The suitability of this IAE-LC–MS assay for analyzing other biosamples was checked by a partial validation of plasma analysis.

2. Experimental

2.1. Chemicals and reagents

α- and β-Amanitin were obtained from Sigma-Aldrich (Deisenhofen, Germany). γ-Amanitin methyl ether was a kind gift of Prof. Dr. Heinz Faulstich, Max-Planck-Institute for Cell Biology, Ladenburg, Germany. Methanol, water (both HPLC grade), and all other chemicals (analytical grade) were obtained from E. Merck (Darmstadt, Germany). HiTrap rProtein A 1 ml columns, CNBr-activated Sepharose 4B and emptied PD10 15 ml columns were obtained from Pharmacia Biotech (Freiburg, Germany). Ultrafree-15 centrifugal filter devices with Biomax-50 membrane were obtained from Millipore (Eschborn, Germany). The syringe filters FP030/2 with a pore size of 0.45 μm for sterile filtration were obtained from Schleicher und Schuell (Dassel, Germany).

2.2. Biosamples

Blank urine samples were collected from healthy volunteers. Authentic urine samples from intoxication cases were submitted to our lab for diagnosis of

an intoxication with amanita mushrooms. Pooled blank plasma samples were obtained from a local blood bank. Authentic plasma samples from intoxication cases were not available.

2.3. Amanitin radioimmunoassay determination

For RIA determinations, the amanitin RIA kit (article no: RK-AM 1) of DPC Biermann (Bad Nauheim, Germany) was used according to the manufacturers manual [15]. The cross reactivity values for α-amanitin are 100% and for β-amanitin 44%.

2.4. Generation, purification and quantification of antibodies

According to Kirchner and Faulstich [16], a β-amanitin-fetuin conjugate was used for immunization of two rabbits. Antiserum was collected and purified in 1-ml-fractions with HiTrap rProtein A columns according to the manufacturer's instructions and the manual by Harlow and Lane [17]. The immunoglobuline G (IgG) containing eluate was concentrated by ultracentrifugation (three times 30 min at 1375 *g*). Finally, the IgG concentration was determined with the Bio-Rad protein assay according to Bradford [18] using bovine IgG as protein standard. About 4 mg of total IgG were received per ml of rabbit serum.

2.5. Preparation of IAE columns

The antibodies were coupled to CNBr-activated Sepharose 4B according to the instructions of the Pharmacia manual: 45 mg of antibody proteins were covalently bound to 14 ml of Sepharose gel [19]. Two milliliter aliquots of gel were filled in emptied PD10 columns. So, a batch of seven identical IAE columns was produced containing 6.4 mg IgG per column. The columns were stored at 4°C in 0.05 *M* mono potassium phosphate buffer (pH 7.4, phosphate buffer) containing 0.02% of sodium azide.

2.6. IAE procedure

In order to remove the sodium azide, the storage buffer was drained off and the IAE columns were washed twice with 10 ml each of phosphate buffer. Urine samples were filtered with a syringe filter of 0.45 μm pore size to prevent contamination of the immobilized antibodies. One hundred microliters of the internal standard (I.S.) solution (1.25 μg/ml γ-amanitin methyl ether) were added to 5 ml of sample (final concentration of the I.S.: 25 ng/ml). This sample was diluted with 4 ml of phosphate buffer to obtain a pH of 7.4 and the column was capped. After 15 min of incubation in an end-over-end shaker (Reax 2, Heidolph, Kelheim, Germany) and elution of the incubation fluid, the column was washed with 10 ml of phosphate buffer and then twice with 10 ml each of water. The column was rinsed with 1 ml of acetone–water (95:5, v/v) which replaced most of the water from the preceding washing step. The bound analytes were eluted with two 15 ml volumes of acetone–methanol (50:50, v/v). The combined eluate was evaporated to dryness at 100°C. The residue was dissolved in 100 μl of HPLC eluent A (cf. Section 2.7.2) and the extract was centrifuged for 2 min at 14 000 rpm. Finally, the sample fluid was transfered into an autosampler vial.

After elution of the analytes, the IAE column was washed with two 15 ml volumes of acetone–methanol (50:50, v/v), equilibrated with 10 ml of water and twice with 10 ml each of phosphate buffer. After addition of sodium azide, the column was stored as given above.

2.7. Liquid chromatography–mass spectrometry

2.7.1. Apparatus

The amanita toxins were separated and identified in biosamples using a Hewlett-Packard (HP, Waldbronn, Germany) atmospheric pressure ionization-electrospray (API-ES) LC–MS including an electrospray interface (HP 59987A), an HP MS Engine 5989 Series B and an HP 1050 HPLC system including an autosampler and a 20 μl sample loop. An electrically actuated three-way valve (Linear Model 0203-7090 stop-flow device, Gamma Analysen Technik, Bremerhaven, Germany) was used to connect either a syringe pump (Harvard, South Natick, USA) for MS tuning purposes or the HPLC system to the electrospray chamber. When the valve was closed, the HPLC effluent flew into the waste.

The valve status could be controlled by the HPLC software.

2.7.2. HPLC conditions

In modification to Ref. [12], gradient separation was achieved on an HP ODS Hypersil RP-18 narrowbore column (100×2.1 mm I.D.) with 3 μm particle size with an HP Hypersil guard column. The mobile phase was methanol–ammonium acetate (0.01 *M*, adjusted to pH 5 with acetic acid; eluent A (10:90, v/v) and eluent B (70:30, v/v)). Before use, the mixture was degassed for 30 min in an ultrasonic bath. During use, the mobile phase was degassed with helium. Until the beginning of the analysis, the HPLC system was flushed with eluent A. The gradient, the flow-rate and the valve status were programmed according to the timetable given in Table 1. After 11.5 min, the HPLC column was re-equilibrated with eluent A, and the autosampler began with the next injection. UV detection was performed at 302 nm with a HP 1050 variable wavelength detector.

2.7.3. Electrospray conditions

The MS conditions were as follows: drying gas (3000 ml/min, 300°C) and nebulizing gas (600 ml/min), nitrogen; capillary exit voltage, 200 V; needle voltage, −5800 V; positive single ion monitoring (SIM) mode with time windows according to the retention times of the analytes. For LC–MS determination in urine or plasma, the following time windows and diagnostic ions were used: ions *m/z* 920, 921, 922 for β-amanitin from 3.4 to 4.0 min; ions *m/z* 919, 920, 921 for α-amanitin from 4.0 to 5.0 min; ions *m/z* 917, 918, 919 for γ-amanitin methyl ether from 5.0 to 6.0 min. The HPLC effluent entered the electrospray chamber only in the time window between 3.4 and 6.0 min.

Tuning of the MS was performed in three steps: first tuning with an erythromycin solution (100 μg/ml of methanol–water (1:1; v/v), tuning ions *m/z* 558, 716 and 734). Fine tuning with a β-amanitin solution (5 μg/ml of mobile phase, tuning ion *m/z* 920). These two tuning steps were done using the syringe pump at a flow-rate of 50 μl/min. Last performance check was done by injection of a standard solution of α- and β-amanitin into the LC–MS (5 μg/ml of both, α- and β-amanitin, including 1.25 μg/ml of the I.S.).

2.8. Assay validation for urine analysis

The IAE–LC–MS assay was validated for the detection of α- and β-amanitin in urine according to the criteria required by Lindner and Wainer [14]. From the batch of seven identical IAE columns, five were used for the validation experiments and two were stored at 4°C.

2.8.1. Preparation of analytical standards, calibration standards and control samples

Stock solutions of α- or β-amanitin, respectively, were prepared independently in methanol (100 μg/ml) from separate weighings. Portions of these stock solutions were diluted with HPLC eluent A to prepare the analytical standard solutions that were used to spike urine for preparation of the calibration standards and quality controls. Calibration standards (5–75 ng/ml) and quality control samples (5–75 ng/ml) of α- and β-amanitin were prepared using pooled blank urine from the independently prepared analytical standard solutions. All solutions were stored at 4°C.

2.8.2. Peak purity and selectivity

Ten blank urine samples from healthy volunteers were analyzed for peaks to interfere with the detection of the analytes or the I.S. In order to examine

Table 1
Timetable of the HPLC gradient. Indicated are the time of the events in (min), the percentage of HPLC eluent B in (%), the flow-rate in (μl/min) and the status of the connection valve between HPLC column and electrospray chamber

Time (min)	0.0	3.0	3.0	3.3	5.0	5.0	6.0	6.0	9.5	9.5
Part of eluent B (%)	100	100	100	100	100	100	100	0	0	0
Flow-rate (μl/min)	350	350	50	50	50	150	150	460	460	350
Valve status	off	off	off	on	on	on	off	off	off	off

simple adsorption to the Sepharose material, a urine sample with 75 ng/ml of α- and β-amanitin and 25 ng/ml of γ-amanitin methyl ether was processed with a blank Sepharose column without immobilized antibodies.

2.8.3. Linearity of calibration

Calibration standards with concentrations of 5, 10, 25, 50 or 75 ng/ml of α- or β-amanitin were extracted and assayed ($n=5$).

2.8.4. Repeatability

Five quality control samples at the low concentration (5 ng/ml, LOW) and five at the high concentration (75 ng/ml; HIGH) of α- or β-amanitin were extracted. The five extracts of each concentration were combined so that a 500 μl sample resulted. Each sample was injected five times within a single sequence and during the course of three consecutive sequences alternately (sequence order: LOW/HIGH/LOW/HIGH/LOW/HIGH).

2.8.5. Accuracy and precision

Spiked control samples ($n=5$) at each of three concentrations (5, 25 or 75 ng/ml of α- or β-amanitin) were assayed against a calibration curve to determine the intra-day accuracy and precision. The concentrations of the analytes were calculated by using a linear regression model and these concentrations were then compared to the nominal concentrations. The calculated values at each concentration were averaged and the percentage bias was calculated to estimate accuracy. The relative standard deviation (RSD) was calculated as a criterion of precision. The inter-day accuracy and precision of the method was assessed from the comparison of the analysis of control samples over three consecutive days in the above mentioned manner.

2.8.6. Stability

The analyte stability for longterm storage was tested by analyzing authentic and spiked samples before and after storage for 6 month at −7°C. The stability of extracted samples was tested by analyzing the extracts before and after storage for 6 months at 4°C. The stability of the IAE columns was tested by comparison of the performance of unused columns that were stored at 4°C for 6 months with that of columns from the same batch that were 50 times in use for the validation experiments.

2.8.7. Limits

For the determination of the limit of detection (LOD) (signal-to-noise ratio 3:1) and the limit of quantification (LOQ) (signal-to-noise ratio 10:1), quality control samples with 2.5 and 5 ng/ml of α- and β-amanitin were assayed ($n=5$). The data from the assay of blank matrices was taken from the selectivity experiments (cf. Section 2.8.2).

2.8.8. Recoveries

Absolute analytical recoveries were tested at the concentration levels of 5 or 75 ng/ml of α- and β-amanitin ($n=5$) and 25 ng/ml of I.S. They were defined as percent peak areas of the corresponding amounts of the analytes spiked to blank urine extracts after extraction versus calibration standards.

2.8.9. Proof of applicability

Five urine samples from authentic amanitins intoxication cases which were tested positive by RIA were assayed with the described method.

2.8.10. Transferability of the immunoaffinity extraction to other biomatrices

In order to examine, whether the IAE was suitable for the extraction of α- and β-amanitin from other biomatrices, the transferability to the processing of plasma was tested as a partial method validation. For that purpose, spiked plasma samples ($n=3$) at each of three concentrations (5, 25 or 75 ng/ml of α- and β-amanitin) were assayed against a calibration curve of urine calibration samples to determine the intra-day accuracy and precision. Then, the selectivity was tested with the analysis of five pooled blank plasma samples. Finally, absolute analytical recoveries were tested with $n=5$ at the concentration level of 75 ng/ml of α- and β-amanitin and were defined as percent peak areas of the corresponding amounts of the analytes spiked into blank plasma extracts after extraction versus calibration standards.

3. Results and discussion

3.1. Generation and purification of antibodies

Amatoxins are haptens, i.e. too small to be immunogenic (M_r about 900), and must therefore be conjugated to a molecule which is large enough to elicit an immune response. But the conjugation of amatoxins to proteins makes them more poisonous than the native toxins, enhancing their uptake into protein-consuming cells [20]. The increased toxicity was not observed when fetuin was used as protein carrier [16]. Using such a conjugate for immunization, antibodies were raised in rabbits. By use of Protein A columns, the total amount of serum IgG was separated from the serum proteins.

3.2. Preparation of IAE columns

The use of IAE for sample preparation is becoming increasingly popular as a tool in the analysis of trace amounts of analytes in biosamples [21–24]. For the procedure presented here, a common approach was used involving direct, covalent attachment of the antibodies by reacting free amine groups on the IgG molecules with CNBr-activated Sepharose. The coupling via amine groups is probably the easiest route to immoblization but can give rise to less than optimum activity because of random orientation or denaturation of the immobilized antibodies. We also tried another way of immobilization in testing AffiGelHz from Bio-Rad. This support involves the coupling through carbohydrate residues on the Fc-region of the IgG molecules which results in an oriented binding of the antibodies to the affinity material. But the IAE columns which were produced by this approach did not work.

3.3. IAE procedure

The IAE procedure was a variation of the method used by Bagnati et al. [25]. An incubation time of 5 min was too short for the used antibodies to bind the analytes completely. Therefore, the incubation time had to be increased up to 15 min. The eluent acetone–water (95:5, v/v) was suitable to desorb a high percentage of the bound analytes, but complete desorption from our antibodies was only possible with acetone–methanol (50:50, v/v). A relatively high elution volume was chosen to save additional washing steps. After elution with 2 times 15 ml, the column was ready for next analysis. As the extracts were quite clean, no additional matrix reached the eluate. The time needed for a complete IAE was 45 min.

Using the new IAE procedure, α- and β-amanitin could selectively be extracted from urine. The amount of extracted matrix could remarkably be lowered in comparison to our previous SPE procedure. Fig. 1 shows two LC–UV chromatograms of the same blank urine sample after extraction by SPE [12] or by IAE. The dotted line shows the UV absorbance of the matrix after SPE. The maximum of the signal is 2500 mAU. The solid line shows the amount of matrix after IAE with a signal maximum of 25 mAU. This means that the IAE allowed to reduce the influence of the matrix by a factor of 100. Since matrix compounds hampered the ionization of the amatoxins in the ES chamber, a matrix reduction was essential for lowering of the LOD. Using IAE, the abundances of the analytes with and without matrix were the same. Thus, the LOD for both, α- and β-amanitin in urine could be lowered to 2.5 ng/ml.

3.4. LC–MS detection

Although several HPLC methods were published for isocratic or gradient elution of amatoxins [4], they cannot be easily transfered to electrospray LC–MS, where only volatile eluent buffer ingredients can be used. In addition, the composition of the eluent should have a high percentage of organic phase, and the flow-rates should be in the range of 100 μl/min for optimal performance (the lower, the better). With these prerequisites in mind, it is easy to understand our rather complicated HPLC gradient program (cf. Table 1). The analytes were flushed over the column at a high flow-rate with the valve between HPLC column and electrospray chamber being closed. When the analytes are leaving the column towards the electrospray, the valve is opened. At 3.0 min, the flow-rate is lowered to 50 μl/min to have an optimal performance for detection. The I.S. has a longer retention time than the analytes. By increasing the flow-rate to 150 μl/min, the total runtime could be

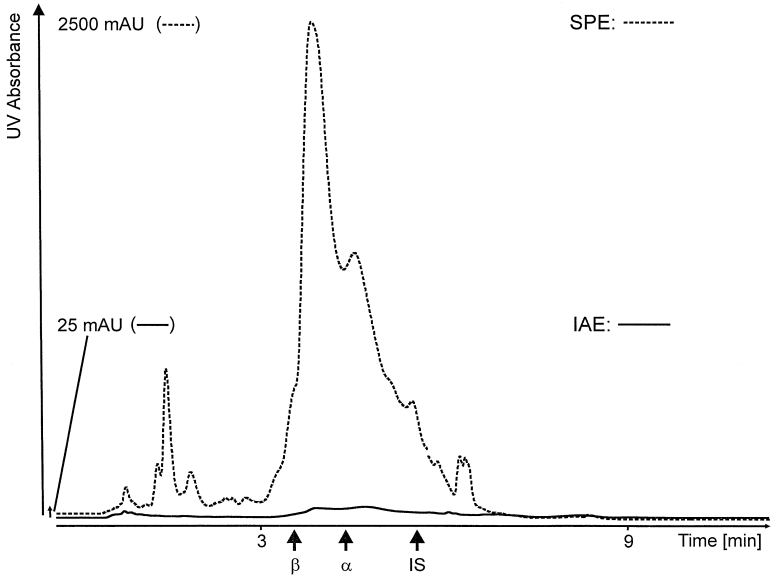


Fig. 1. LC–UV chromatograms of a blank urine sample after extraction by SPE (dotted line) or by IAE (solid line). The arrows mark the retention times of α-amanitin (α), β-amanitin (β) and the I.S. (Chromatographic conditions: gradient separation on HP ODS Hypersil RP-18 narrowbore column (100×2.1 mm I.D., 3 μm); mobile phase, methanol–ammonium acetate (0.01 *M*) 10:90 (v/v) and 70:30 (v/v); UV detection at 302 nm.).

shortened without remarkable loss in performance. After the detection, the valve was closed at 6.0 min and the column was re-equilibrated.

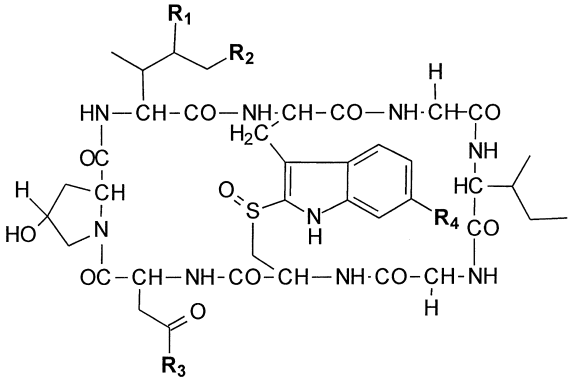
A runtime of 12 min for the analysis of one sample including re-equilibration of the HPLC column could be reached, which is only 30% of the time needed with our previous isocratic method. Thus, 5 samples could be assayed per hour. Keeping in mind, that the IAE needed 45 min, and knowing that there was no analyte carry-over between two consecutive LC–MS runs, extracts produced beforehand for establishing a four-point calibration curve could be injected during the extraction time. Together with the sample run, the complete analysis needed 60 min, which is 30 min faster than the RIA analysis.

The indicated SIM time windows and SIM diagnostic ions could be inquired by analysis of the pure substances in the full scan mode. In Fig. 2, the structures, empirical formulae and molecular masses of α- and β-amanitin and the I.S. are shown. The described LC–MS method was also suitable for the detection of amatoxins and phallotoxins in mushroom material after a simple and fast aqueous extraction [26].

3.5. Validation data

Mass fragmentograms of a blank urine sample are presented in Fig. 3. No endogenous peaks disturbed significantly the integration of the analytes or the I.S. Simple adsorption of amanitins on sepharose could be excluded.

The calibration curve for α- and β-amanitin was linear from 5 to 75 ng/ml with mean r^2 values of 0.9973 and 0.9907, respectively. The lower to middle and the middle to upper point of slope deviated less than 5% from the overall slope. The repeatability was examined in the indicated manner (cf. Section 2.8.4) and with $n=15$, the RSD values for α- and β-amanitin were 7.0 and 7.6% for the low control and 6.1 and 8.1% for the high control. The intra- and inter-day accuracy and precision data for the quantification of the quality control samples are shown in Tables 2 and 3. The processing of authentic and



α-Amanitin
$R_{1,2,4}$=OH, R_3=NH_2
$C_{39}H_{54}N_{10}O_{14}S$
Mass: 918 u

β-Amanitin
$R_{1,2,3,4}$=OH
$C_{39}H_{53}N_9O_{15}S$
Mass: 919 u

γ-Amanitin methyl ether
R_1=OH, R_2=H, R_3=NH_2, R_4=OCH_3
$C_{40}H_{56}N_{10}O_{13}S$
Mass: 916 u

Fig. 2. Structures, empirical formulae, molecular masses of α- and β-amanitin and the internal standard γ-amanitin methyl ether.

spiked urine samples which were stored at −7°C for 6 months and were thawn and frozen several times showed no differences between the initial and the actual determination. The concentrations determined by LC–MS were in congruence with the RIA results (cf. determination of authentic samples below). Immunoaffinity extracts were stable for at least 6 months when stored at 4°C.

The immobilized antibodies were stable without use for at least 6 months when stored at 4°C. Setting the performance of a new IAE column for extraction of a quality sample (50 ng/ml of α- and β-amanitin) as 100%, the used IAE columns (50 extractions) had a performance of 96.7±7.3% and 98.1±10.7%. This is in accordance with the literature where IAE columns were found being able to perform up to 100 extractions without significant loss of activity [25]. The LOD was 2.5 ng/ml with a signal-to-noise ratio (S/N) of 3:1, and the LOQ (S/N 10:1) was 5 ng/ml which was the lowest concentration used for the calibration curve (cf. Fig. 3). The absolute recoveries (mean±RSD, n=5) of α- and β-amanitin were 62.5±13.9% and 57.5±11.5% for the low quality control and 61.4±4.6% and 56.6±6.5% for the high quality control. The absolute recovery (mean±RSD, n=5) for γ-amanitin methyl ether at 25 ng/ml was 60.3±2.8%. The recoveries were relatively low, but sufficient for quantitative purposes since they were reproducible.

As shown in Table 4, the analysis of five authentic urine samples from patients intoxicated by amatoxin containing mushrooms showed a good correlation between the results from RIA analyses and the IAE–LC–MS assay taking into consideration the different cross reactivity values for α- and β-amanitin. The determined concentrations ranged from 12 to 66 ng/ml amatoxins, which is in accordance with published data. The amatoxin concentrations in 93 urine samples tested positive by RIA ranged between 10 to 100 ng/ml in most cases [27]. The calibration range of our method covers the concentration range which is to be expected after ingestion of amatoxin containing mushrooms. In Fig. 4, corresponding mass fragmentograms of an authentic urine sample after IAE are given indicating 16 ng/ml of β-amanitin and 13 ng/ml of α-amanitin (RIA value 20 ng/ml amatoxins).

The comparison of our method to the validated method of Defendenti et al. [7] for the determination of α-amanitin in urine shows the following advantages: our method works with only 5 ml instead of 10 ml sample volume and is able to determine α- and β-amanitin also in plasma; the LOQ of our method is 5 ng/ml instead of 10 ng/ml. The LOD was 2 ng/ml for the Defendenti method, but it was assayed as pure substance standard and not under matrix influence. As mentioned above, the electrochemical detection is not as specific as the MS detection. These authors concluded that their method could only be used ''in parallel with the RIA for mutual confirmation of results''. The assay presented in this paper is accurate, specific and sensitive enough to be used as exclusive method for the diagnosis of an intoxication with amatoxins.

As expected, the IAE is transferable to the analysis of other biomatrices. In order to examine the possibility to analyze plasma samples, a partial

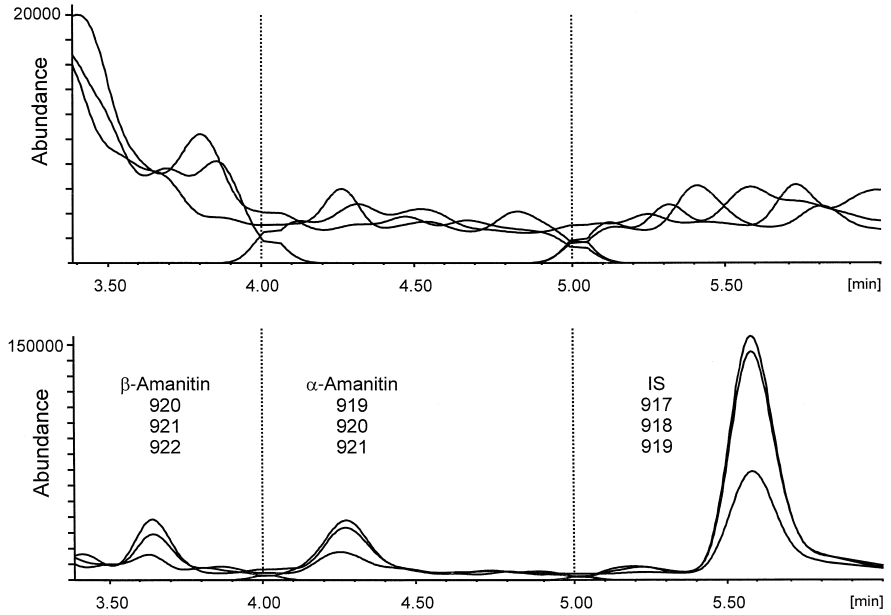


Fig. 3. Merged mass fragmentograms with the given ions of extracts of a blank urine sample (*top*) and of a blank urine sample spiked with 5.0 ng/ml of α- and β-amanitin and 25 ng/ml of γ-amanitin methyl ether (*bottom*). The dotted lines indicate the limits of the different time windows for SIM analysis (3.4–4 min: *m/z* 920, 921, 922; 4–5 min: *m/z* 919, 920, 921; 5–6 min: *m/z* 917, 918, 919).

method validation in the described manner was performed (cf. Section 2.8.10). The intra-day accuracy and precision data for the quantification of the plasma quality control samples are shown in Table 5. The analysis of five blank plasma samples showed no endogenous peaks which interfered significantly with the integration of the analytes or the internal standard. The absolute recoveries (mean±RSD, $n=5$) of α- and β-amanitin from plasma were 49.1±3.4% and 52.1±9.0% for the high quality control level.

4. Conclusions

The IAE-LC–MS assay presented here allowed the precise and sensitive determination of α- and

Table 2
Intra-day accuracy and precision of the determination of α- and β-amanitin in urine

Intra-day ($n=5$)	Actual concentration (ng/ml)		Mean calculated concentration (ng/ml)		Precision (%)[a]		Accuracy (%)[b]	
	α	β	α	β	α	β	α	β
Low QC	5	5	5.3	5.2	2.3	3.6	6.0	4.7
Medium QC	25	25	25.7	26.1	2.8	2.2	2.7	4.6
High QC	75	75	75.6	77.0	2.0	3.6	0.8	2.7

[a] Precision=(SD/mean)×100.
[b] Accuracy=((mean calculated concentration−actual concentration)/actual concentration)×100.

Table 3
Inter-day accuracy and precision of the determination of α- and β-amanitin in urine

Inter-day (n=5) 3 days	Actual concentration (ng/ml)		Mean calculated concentration (ng/ml)		Precision (%)[a]		Accuracy (%)[b]	
	α	β	α	β	α	β	α	β
Low QC	5	5	5.7	6.0	19.8	12.1	13.9	19.0
Medium QC	25	25	26.6	24.3	8.0	5.3	6.4	−2.9
High QC	75	75	74.2	78.2	3.9	7.4	−1.0	4.2

[a] Precision=(SD/mean)×100.
[b] Accuracy=((mean calculated concentration−actual concentration)/actual concentration)×100.

Table 4
Analysis of authentic urine samples by RIA and IAE-LC–MS. Corrected sum means the sum of α-amanitin and β-amanitin considering the cross reactivities of the RIA antibodies for α-amanitin (100%) and β-amanitin (44%). The RIA covers both amanitins and should therefore yield this value (brackets indicate, that the given values are outside the linearity range)

Sample	RIA (ng/ml) Amanitins	LC–MS (ng/ml)		
		β-amanitin	α-amanitin	Corrected sum
1	14	16	5	12
2	20	16	13	20
3	27	31	14	28
4	29	23	17	27
5	>60	(89)	27	(66)

β-amanitin in urine, thus allowing the specific diagnosis of an intoxication with amatoxin containing mushrooms throughout the year. The method fulfilled the requirements for a validated assay and was also suitable for analysis of plasma.

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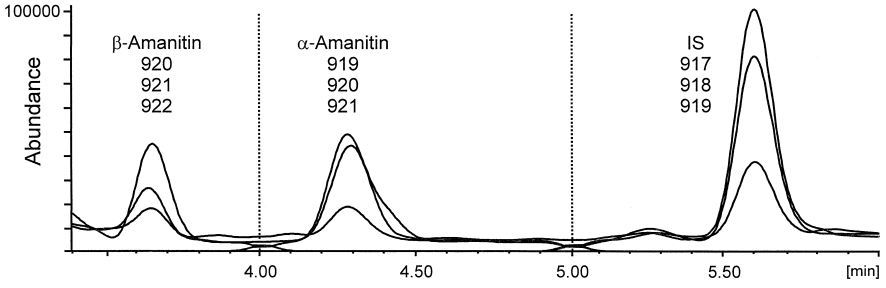

Fig. 4. Merged mass fragmentograms with the given ions of an extract of an authentic urine indicating 16 ng/ml of β-amanitin and 13 ng/ml of α-amanitin; (RIA value 20 ng/ml amatoxins). The dotted lines indicate the limits of the different time windows for SIM analysis (3–4 min: m/z 920, 921, 922; 4–5 min: m/z 919, 920, 921; 5–6 min: m/z 917, 918, 919).

Table 5
Intra-day accuracy and precision of the determination of α- and β-amanitin in plasma

Intra-day (n=3)	Actual concentration (ng/ml)		Mean calculated concentration (ng/ml)		Precision (%)[a]		Accuracy (%)[b]	
	α	β	α	β	α	β	α	β
Low QC	5	5	5.0	5.2	2.5	3.0	−0.1	3.1
Medium QC	25	25	26.6	24.4	0.7	4.7	6.5	−2.4
High QC	75	75	76.1	77.2	1.4	0.8	1.5	2.9

[a] Precision=(SD/mean)×100.
[b] Accuracy=((mean calculated concentration−actual concentration)/actual concentration)×100.

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